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TOXICITY EVALUATION OF CHLORDECONE AND ITS EFFECT ON OXIDATIVE IMBALANCE IN THE CICHLID FISH, *ETROPLUS MACULATUS* (BLOCH)

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ABSTRACT

The aim of the present study was to evaluate the toxicity effect of chlordecone on the reactive oxygen species generation in the Cichlid fish, *Etroplus maculatus*. Chlordecone (0.35 µg/L) was used as the test dose and the fishes were treated for 24, 48 and 96 h maintaining a control group without adding the test chemical. Body weight of the treated fishes remained unchanged throughout the experiments. However, mucous deposition was significantly increased in time-dependent manner which states the defensive mechanism of the exposed fishes to chlordecone. Antioxidant parameters such as superoxide dismutase, catalase, glutathione reductase and the levels of hydrogen peroxide and lipid peroxidation were evaluated in both gill and liver tissues. Statistical analysis reports that chlordecone induced oxidative stress on gill as well as in liver by significant ($p<0.05$) reduction in the activities of antioxidant enzymes with concomitant increase in the levels of hydrogen peroxide and lipid peroxidation. The present study also showed a significant reduction in the marker enzyme, alkaline phosphatase in gill and liver and it could be due to decreased state of inter and intracellular membrane transport and possibly this could be also due to the toxicity of chlordecone. Histopathological observations reveal chlordecone-treated gill with destruction of primary and secondary lamellae, upliftment of gill epithelium and reduction in the number of chloride cells. Similarly exposure to chlordecone critically affected the architecture of hepatocytes with enucleated cells, cytoplasmic vacuolization and hepatic necrosis. Thus toxicity of chlordecone by reducing the activities of antioxidant enzymes resulted in oxidative imbalance in the vital organs as gill and liver of the fish, *Etroplus maculatus*.

KEYWORDS: *Etroplus*, Gill; Liver, ROS, Antioxidant Enzymes, Lipid Peroxidation, Alkaline Phosphatase

INTRODUCTION

Pesticides when used in the vicinity of aquatic ecosystem may enter into the water bodies as a result of spray drift and leaching from the soil, which may exert an adverse effect on aquatic populations including fish. In the assessment of the toxicity of pesticides to fish, studies have been largely restricted to the direct effects of individual compound; however, under field conditions the metabolism and toxicity of pesticides could be modulated by simultaneous exposure to combinations of pesticides with other pollutants. Such pollutants are of great concern to the human population as it directly or indirectly enter the humans through food chain. In the biological system such pollutants cause detrimental effect on various systems of several vital organs.

In the present study one such environmental pollutant preferred is chlordecone (Kepone). Chlordecone is a synthetic chlorinated organic compound, which has been mainly used as an agricultural insecticide, miticide and fungicide. It is widely applied in the control of the banana root borer, applied on non-fruit-bearing citrus trees to control rust mites, control of wireworms in tobacco fields, control of apple scab and powdery mildew, control of the grass mole cricket, and control of slugs, snails, and fire ants. The use of chlordecone was prohibited in Western countries, but it is still used in India. Chlordecone is resistant to degradation in the environment and has a high potential for bioaccumulation in fish and

other aquatic organisms (ATSDR, 1995). Chlordecone has been shown to be eliminated more slowly from the liver as compared with other tissues in rats (Belfiore et al., 2007). Chlordecone absorption in humans has been demonstrated by the measurement of chlordecone concentrations in blood, subcutaneous fat, and other body fluids and tissues following subchronic occupational exposure, presumably through ingestion, inhalation, and dermal contact (Taylor, 1982; Cohn et al., 1978). Animal studies suggest that chlordecone is absorbed only to a limited extent through the skin (Heatherington et al., 1998). Chlordecone has been shown to cross the placenta and was observed in rat fetal tissues as early as 4 hours after maternal dosing (Kavlock et al., 1980). Chlordecone readily metabolized into chlordecone alcohol by a cytosolic aldo-keto reductase enzyme and eliminated from the body primarily through biliary excretion into feces (Molowa et al., 1986).

Fish are the most widely used non-mammalian vertebrates in risk assessment and regulation of various environmental pollutants. Several fish species are recommended for standard testing of chemicals or environmental samples. Among them, *Etroplus maculatus* (Bloch) has special characteristics, which expedite its use as a model organism in the present study. *Etroplus*, a fresh water Cichlid fish popularly known as orange chromide, commonly found in rivers, streams and canals of India. *Etroplus* is an endemic species of southern India and are very common to lotic water system has been known to pose severe health risk when exposed to such toxicants. Most studies on the effects of chlordecone are confined to mammals and other fish populations. Several studies also focused on the estrogenic properties of chlordecone and no information is available on the antioxidant effect of chlordecone on the euryhaline fish, *Etroplus*. The present study is therefore designed to evaluate the role of chlordecone on the antioxidant system as well as to study the toxicity due to chlordecone could be the cause of oxidative imbalance in liver and gill of the fish.

MATERIAL AND METHODS

Collection and Maintenance of Animal

The Cichlid fish, *Etroplus maculatus* weighing 7 ± 1 g and length 7 ± 1.5 cm were collected from a fish farm, KKF Nursery, Manjeri, Vaniyambalam. Fishes were acclimatized to the laboratory conditions prior to experiments and were exposed with constant supply of water and good lighting system. They were maintained in well-aerated tubs (40 L capacity), which was dechlorinated and sustained with fresh water flow and waste water discharge.

Preliminary Tests

The physico-chemical features of the tap water were estimated as per APHA (1998). Water temperature in the test ranged from 28 ± 2°C during the experiment, oxygen saturation of water ranged between 70 and 100 %, pH is 6.5 to 7.5 which were monitored using a standardized procedures. The LC_{50} values in the respective time intervals were determined by probit analysis, with a confident limit of 5 % level (Finney, 1971). Preliminary tests were conducted to provide guidance on range of concentration of pesticide to use in the bioassay. The specimens were not fed a day prior to and during toxicity tests to reduce faecal and excess food contaminating the test solution. Five specimens were placed in each tub of replicates so that ten fishes were maintained in each test and aerated using tubed motorized pumps. Monofilament netting was used to cover the tanks to prevent the specimens from jumping out of test solutions. The behaviour of specimens was observed and death was also recorded throughout the study.

Evaluation of Median Lethal Concentration (LC_{50})

The concentration of the pollutant at which 50 percentage of the test animals dies during a specific period or the concentration lethal to one half of the test population is referred to as median lethal concentration (LC_{50}) or median tolerance limit. For determining LC_{50} concentration separate circular plastic tubs of 40 L of water capacity were taken and

different concentrations of chlordecone were added. Then, 10 fish were introduced into each tub. A control tub with 40 L of water and 10 fishes were also maintained (no toxicant). The lethal concentration for 50 % killing (LC_{50}) values was computed on the basis of probit analysis (Finney, 1971) for 96 h, which was 35 µg/ L. One-tenth of the dosage (0.35 µg/ L) of chlordecone was chosen in the present study.

Chemicals

Technical grade organophosphate insecticide, chlordecone (Kepone, decachlorooctahydro-1,3,4-metheno-2H-cyclobuta[cd]-pentalen-2-one, 99.9% pure) was obtained from Supelco, USA. Malondialdehyde, NADPH and glutathione oxidized were obtained from SISCO Research Laboratories, Mumbai, India. Thiobarbituric acid and pyrogallol were obtained from Himedia Laboratories, Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

Treatments

There were four groups, three tanks with toxicant doses and a control tank. Single dose with three durations were used in present study. Ten fish specimens were used for every test and also in control. The first group of fish was maintained in pesticide free water and used as control and the second group was treated with chlordecone at 0.35 µg/ L for 24 h; the third group was treated with chlordecone at 0.35 µg/ L for 48 h and the fourth group was also treated with chlordecone at 0.35 µg/ L for 96 h. Biochemical estimation of liver and gill was performed at the end of 24, 48 and 96 hours of treatment, at the same time the control group was also maintained.

Killing of Animals

The fish was caught very gently using a small dip net, one at a time with least disturbance. At the end of each exposure time, fishes were decapitated. Liver and gill were dissected and stored at 4ºC until the analyses were performed.

Tissue Processing and Biochemical Analysis

A 1% (w/ v) homogenate of liver and gill was prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 8000 ***g*** for 15 min at 4°C to obtain the supernatant, which was then used for the biochemical analyses.

Protein was estimated by the method of Lowry et al (1951) with BSA as the standard. Activity of superoxide dismutase was estimated by the method of Marklund and Marklund, 1974. The activity of catalase was measured by the method of Claiborne, 1985. Glutathione reductase was assayed by the method of Carlberg and Mannervik (1985). Levels of hydrogen peroxide generation were assayed by the method of Pick and Keisari (1981), levels of lipid peroxidation were measured by the method of Ohkawa et al., 1979. Alkaline phosphatase was measured as described by Bessey et al., 1946.

Histology of Tissues

Liver and gill tissues were collected by sacrificing the fish. The tissue was fixed in 10 % formalin for 24 hours. Tissue was dehydrated in ascending grades of alcohol and was cleared in xylene until they became translucent. Tissue was transferred to molten paraffin wax for 1 hour to remove xylene completely and then impregnated with wax. Then the blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns. The sections were stained with haematoxylin and eosin and mounted in DPx. The structural alterations were observed under light microscope in the sections of liver and gill and were compared with those of control tissues. Photomicrographs were taken using Cannon shot

camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

Statistical Analyses

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 19.0. Differences were considered to be significant at $p<0.05$ against control group. Data are presented as mean ± SD for ten animals per group. All biochemical estimations were carried out in duplicate.

RESULTS

Chlordecone did not cause any significant changes in the body weight of the animals (Figure 1). However, the mucous deposition was increased (Figure 2) significantly ($p<0.05$) in time-dependent manner. The weights of gill and liver decreased significantly after 96 h of chlordecone exposure (Figure 3). The activities of superoxide dismutase and catalase decreased in time-dependent manner in gill and liver tissues (Figure 4 and 5). The activity of glutathione reductase decreased significantly in all treatment groups (Figure 6) with associated increase in the levels of hydrogen peroxide and lipid peroxidation (Figures. 7 and 8) in the liver as well as gill of fishes as compared with the control groups. A significant ($p<0.05$) decrease in the activity of alkaline phosphatase in both the liver and gill was observed after chlordecone treatment (Figure 9).

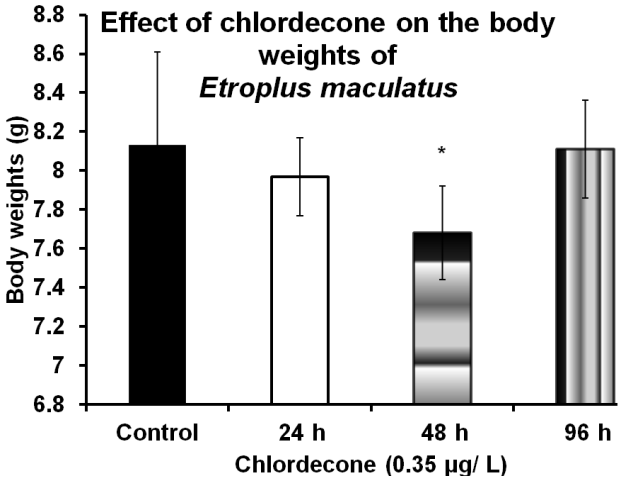


Figure 1

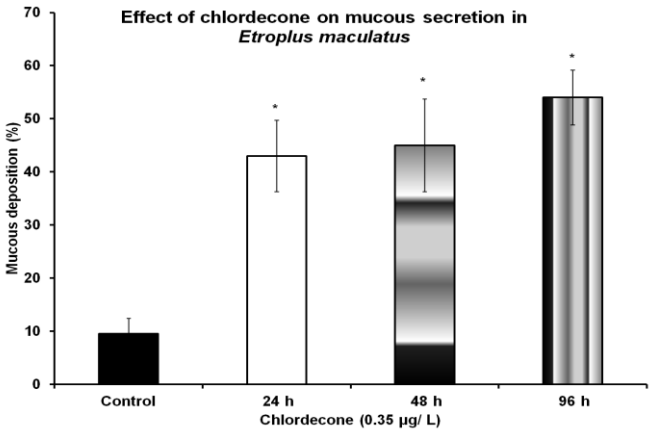


Figure 2

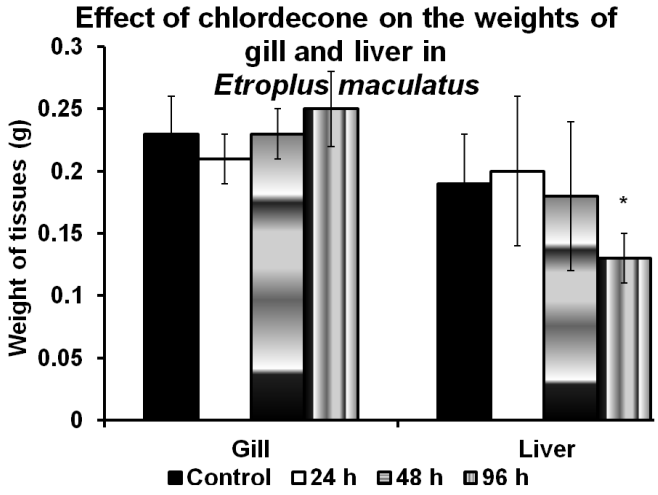


Figure 3

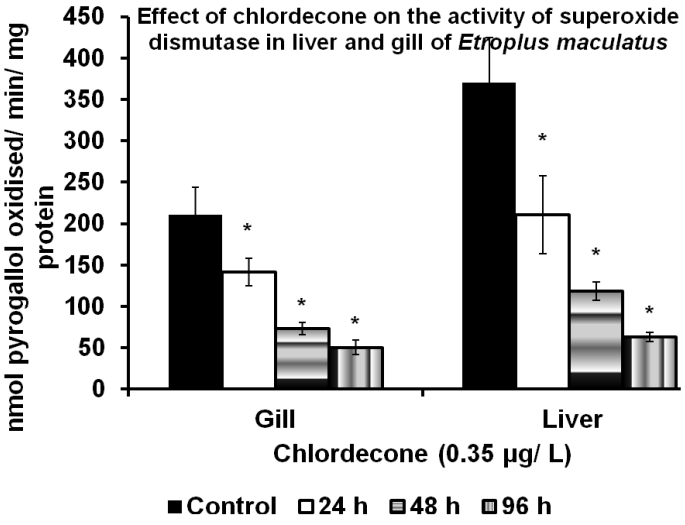


Figure 4

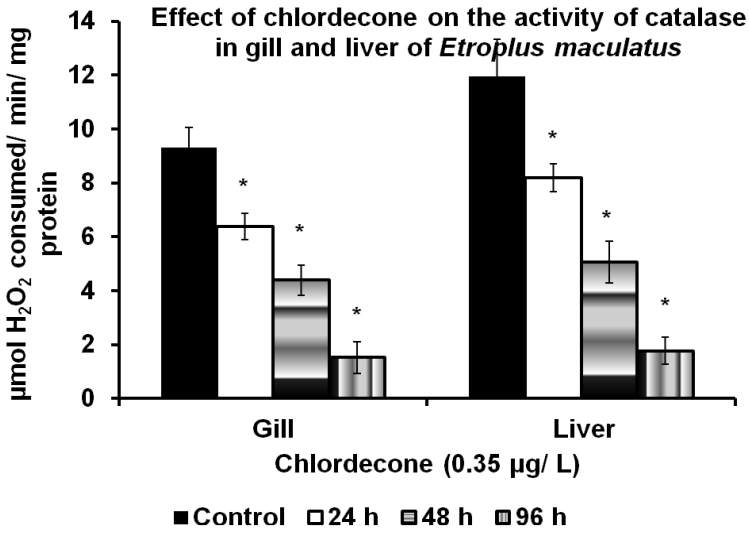
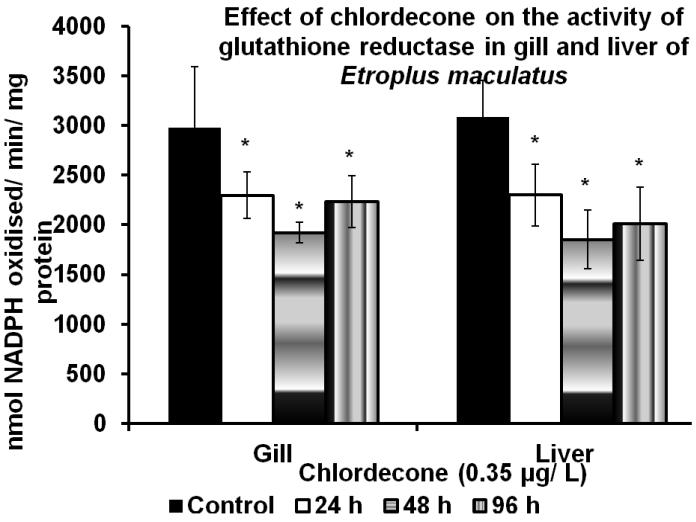


Figure 5



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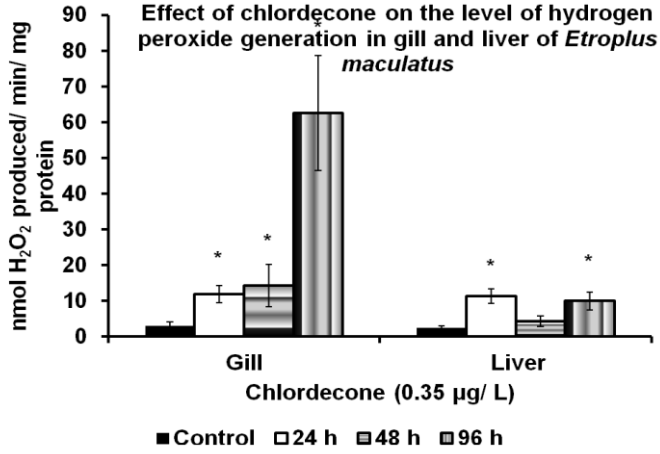

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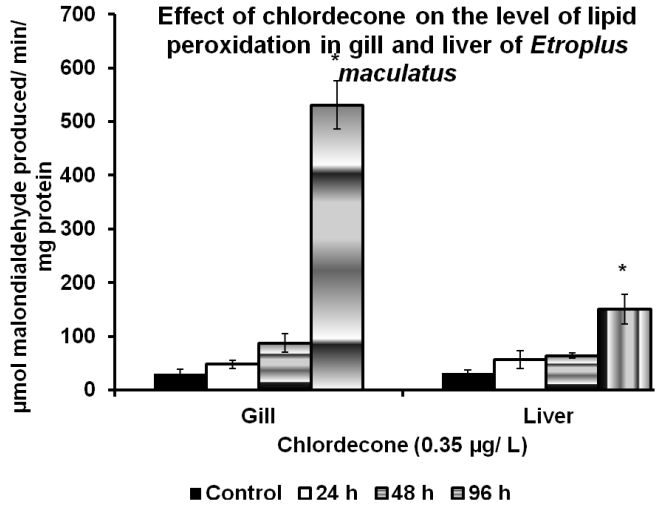

Figure 8

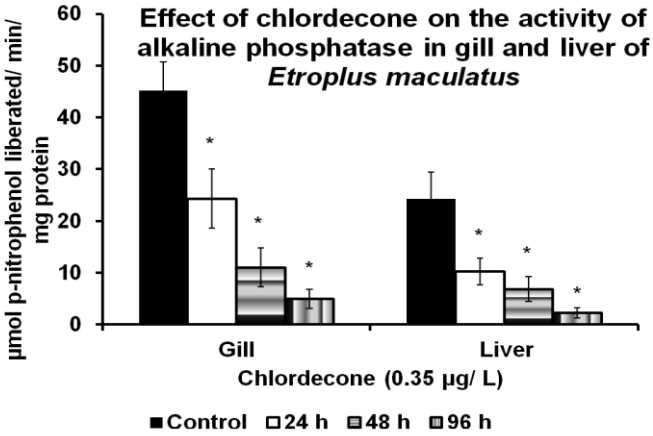

Figure 9

Histopathology of control liver showed normal hepatocytes with granular cytoplasm and spherical nucleus (Figure 10). Chlordecone showed disruption in normal architecture of hepatocytes which is revealed by disorganized hepatocytes in 24 h treatment (Figure 11), cytoplasmic vacuolization and enucleated hepatocytes at 48 h (Figure 12) and complete necrosis and disorientation of hepatocyte with spherical to oval nucleus at 96 h (Figures 13a and 13b).

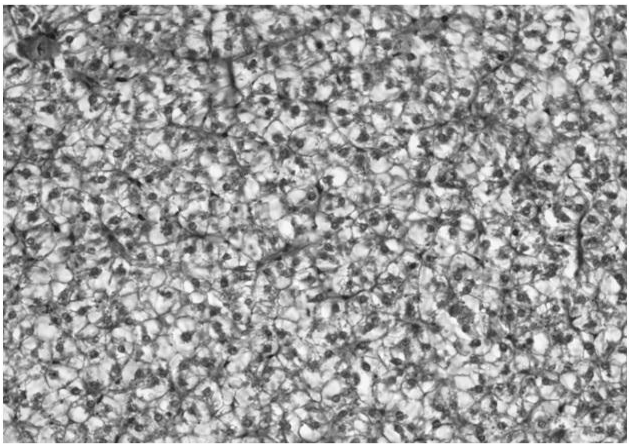

Figure 10: Photomicrograph Showing Histology of Liver of the Control Fish (X 400 Magnification)

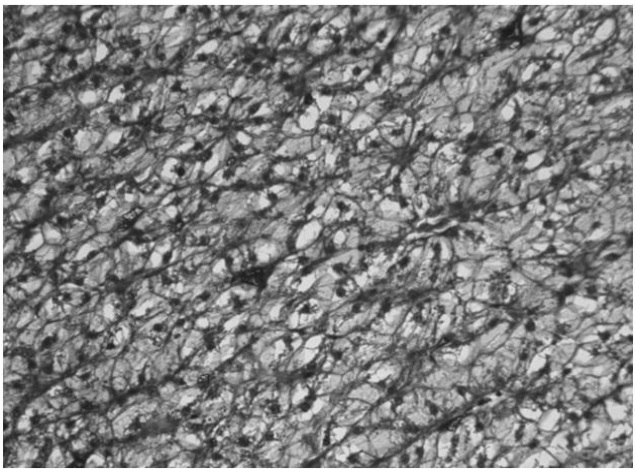

Figure 11: Photomicrograph Showing Histology of Liver - 24 h of Chlordecone Treatment (X 400 Magnification) Showing Disorganized Hepatocytes

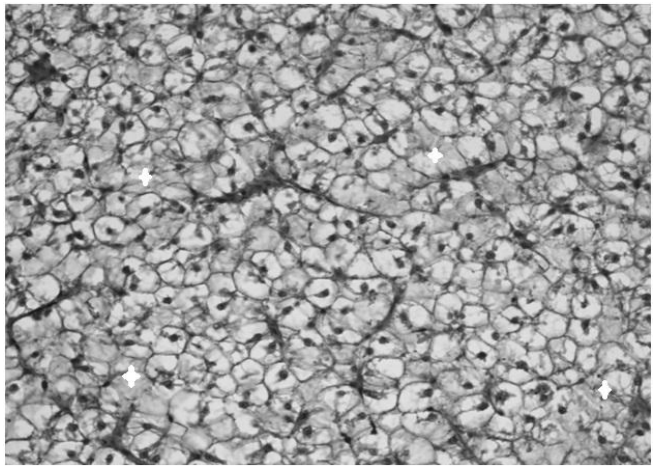

Figure 12: Photomicrograph Showing Histology of Liver - 48 h of Chlordecone Treatment (X 400 Magnification) Showing Cytoplasmic Vacuolization; Asterisks (*) Represents Enucleated Cells

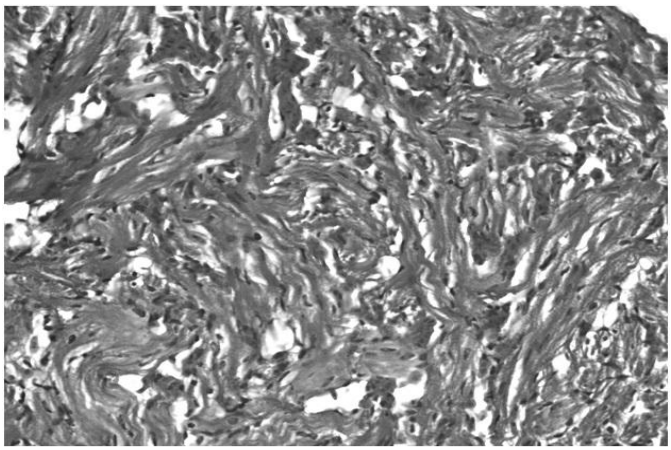

Figure 13a: Photomicrograph Showing Histology of Liver - 96 h of Chlordecone Treatment (X 400 Magnification) Showing Disorientation of Hepatocyte with Spherical to Oval Nucleus

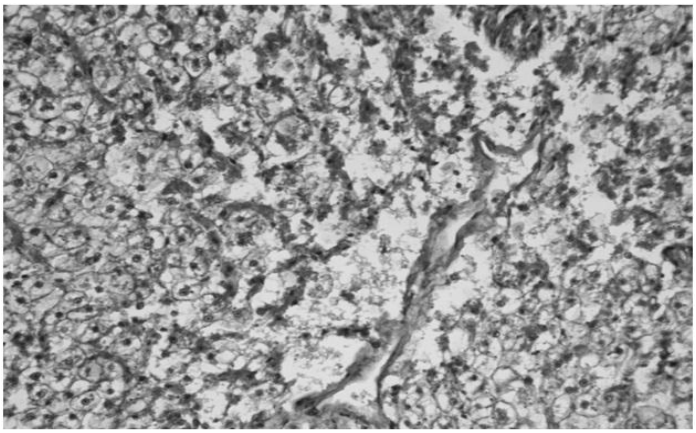

Figure 13b: Photomicrograph Showing Histology of Liver - 96 h of Chlordecone Treatment (X 400 Magnification) Showing Complete Necrosis of Hepatocytes

Histopathological observation of gill from control group showed that the gill lamellae are separated from each other (Figure 14) whereas exposure to chlordecone seriously affected gill histology which is observed as primary and secondary lamellar fusion at 24 h of exposure (Figure 15). In 48 h of chlordecone treatment the upliftment of lamellar epithelium, decrease in chloride cells and hyperplasia at distal end of secondary lamellar epithelium was observed (Figures 16a and 16b). Chlordecone treatment demonstrated epithelial lifting of secondary lamellae, leucocyte infiltration and absence of chloride cells at 96 h of exposure (Figures 17a and 17b).

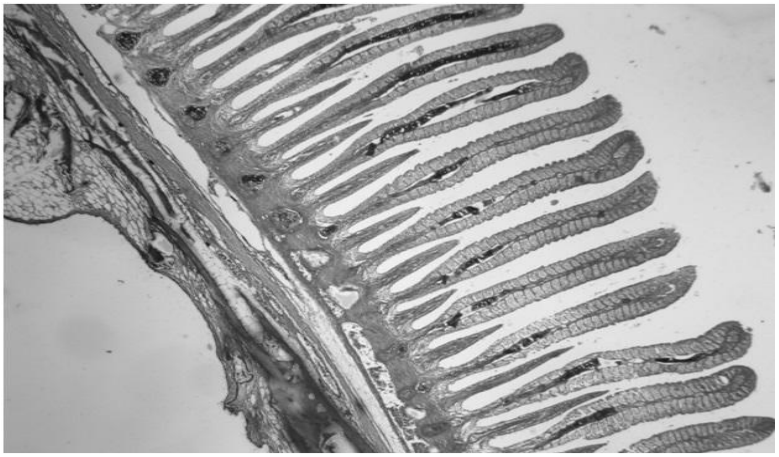

Figure 14: **Photomicrograph Showing Histology of Gill of the Control Fish (X 40 Magnification)**

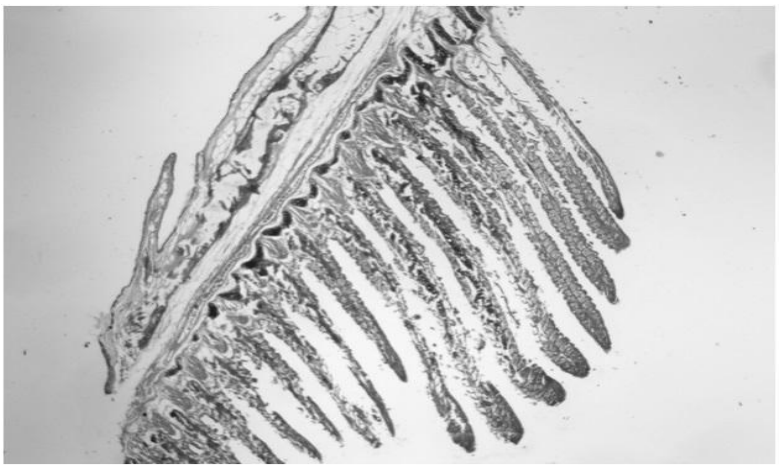

Figure 15: Photomicrograph Showing Histology of Gill - 24 h of Chlordecone Treatment (X 40 Magnification) Showing Primary and Secondary Gill Lamellar Fusion

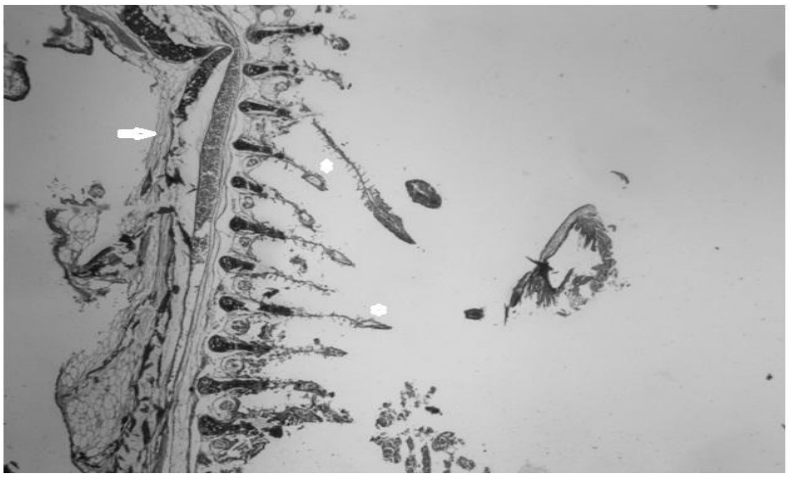

Figure 16a: Photomicrograph Showing Histology of Gill - 48 h of Chlordecone Treatment (X 40 Magnification) Arrow Represents Upliftment of Lamellar Epithelium;Asterisks (*) Represents Hyperplasia at Distal End of Secondary Lamellar Epithelium

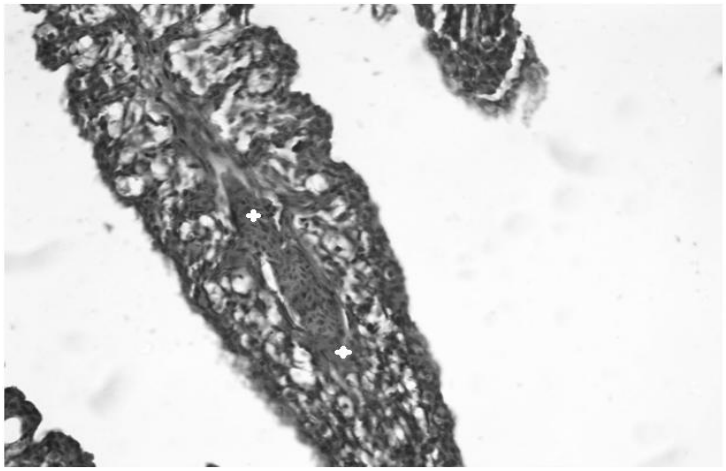

Figure 16b: Photomicrograph Showing Histology of Gill - 48 h of Chlordecone Treatment (X 400 Magnification) Arrow Represents Decrease in Chloride Cells

Figure 17a: Photomicrograph showing histology of gill - 96 h of chlordecone treatment (X 400 magnification) Arrow represents absence of chloride cells

Figure 17b: Photomicrograph Showing Histology of Gill - 96 h of Chlordecone Treatment (X 400 Magnification) Arrow Represents Leucocyte Infiltration

DISCUSSIONS

All living organisms have the ability to acclimatize themselves to the changes in the environment such as temperature, humidity, oxygen supply or to the exposure of toxicants. In some cases, especially in fish they may resist the change for a period of time, but will eventually succumb as a result of the inability to adapt to the change. The effect of one

of the toxicants, chlordecone was evaluated in the present study on some parameters associated with the physiology, biochemistry and histopathological features of fish. The hypothesis that was set in the present study is to evaluate if the toxicity of chlordecone at tolerable sublethal concentration (0.35 µg/ L) could be the cause of oxidative imbalance in the vital organs as liver and gill of the fish, *Etroplus maculatus*. In order to examine the sequence of physiological changes associated with chlordecone toxicity, the body weight, weights of gill and liver, mucous secretion, and the biochemical parameters as antioxidant enzyme systems and histopathological changes of gill and liver were observed at 24, 48 and 96 h intervals.

In the present study chlordecone treatment showed a significant increase in the mucous secretion in time-dependent manner without significant changes in the body weights when compared with that of control groups. Mucous cells are considered efficient in seizing the toxic agents and thus help in the prevention of the entrance of these agents into the gills (Perry and Laurent, 1993). Hypersecretion of mucous may be the consequence of a chronic defensive mechanism of the fish against the exposure to the environmental toxicant chlordecone. The weights of gill and liver was significantly decreased in all treatment groups which indicate the toxicity of chlordecone on fish and this could be possibly due to atrophy of gill lamellae or necrosis of hepatocytes which is evidenced by the histopathological observations.

Histopathological biomarkers can be the indicators of the effects of various anthropogenic pollutants on animals and are a reflection of the overall health of the entire population in the ecosystem. The alterations in cells and tissues in vertebrate fish are recurrently used as biomarkers in many studies. Histopathological biomarkers are closely related to other biomarkers of stress since many pollutants have to undergo metabolic activation in order to be able to provoke cellular change in the affected organism. Therefore histopathological observations are acknowledged as a fast and valid method to determine the damages caused by exposure to different pollutants in fishes (Arellano et al., 2001). However, fish exposed to chlordecone showed several histological alterations, namely primary and secondary lamellar fusion at 24 h of exposure, upliftment of lamellar epithelium, decrease in chloride cells and hyperplasia at distal end of secondary lamellar epithelium was observed after 48 h of exposure. Chlordecone treatment demonstrated epithelial lifting of secondary lamellae, leucocyte infiltration and absence of chloride cells at 96 h. The lifting of gill lamellar epithelium is probably induced by the incidence of severe edema. Edema or hyperplasia with lifting of lamellar epithelium could serve as a mechanism of defense, because separation of epithelial lamellae increases the distance across which waterborne pollutants must diffuse to reach the bloodstream (Arellano et al., 1999). However, these results also were found in fish exposed to other pollutants. The edema, epithelial lifting as well as lamellar fusion also are defensive mechanisms that reduce the branchial superficial area in contact with the external milieu (Chitra et al., 2012).

Liver plays a key role in the metabolism and biochemical transformations of pollutants from the environment, which inevitably reflects on its integrity by creating lesions and other histopathological alterations of the liver parenchyma or the bile duct (Roberts, 1978). In the present study exposure to chlordecone also caused alterations in normal architecture of liver histology in all treatment groups that are evidenced by disorganized hepatocytes, cytoplasmic vacuolization, enucleated hepatocytes, necrosis and disorientation of hepatocyte with spherical to oval nucleus. Cytoplasmic vacuolization in hepatocytes commonly occurs in fish as a histopathological response to aquatic pollutants. Anomalies such as irregular shaped hepatocytes, cytoplasmic vacuolization and nucleus in a lateral position, close to the cell membrane, were also described in the siluriform *Corydoras paleatus* contaminated by organophosphate pesticides (Fanta et al., 2003). Vacuoles in the cytoplasm of the hepatocytes can contain lipids and glycogen, which are related to the normal metabolic function of the liver. Depletion of the glycogen in the hepatocytes is usually found in stressed animals, because the glycogen acts as a reserve of glucose to supply the higher energetic demand occurring in such situations

(Hinton and Laurén, 1990).

Several data indicate that the pollution toxicity in aquatic organisms may be associated with increased production of reactive oxygen species, leading to oxidative stress. In the aquatic environment, despite the reduction in the antioxidant defense systems, increased levels of oxidative damage may occur in organisms exposed to contaminants that stimulate the production of reactive oxygen species. Thus the lesions in liver or gill morphology could lead to functional alterations and interference in fundamental process such as maintenance of osmoregulation and antioxidant defense of gills or hepatocytes.

Reactive oxygen species generation can lead to cell death; however, cells possess a variety of defensive mechanisms including cell-cycle delay, the induction of enzymes such as catalases, peroxidases, and superoxide dismutases, and the synthesis of antioxidants as glutathione, vitamins C and E. But after certain extent of toxicant resistance the animal could not withstand the stress thereby the activity of antioxidant enzymes decline and level of lipid peroxidation increases dramatically resulting in oxidative stress. In the present study chlordecone treatment decreased the activities of antioxidant enzymes and showed a concomitant increase in the levels of hydrogen peroxide generation and lipid peroxidative in this manner proving that the animal could not endure the toxic effect of chlordecone and it imbalances the pro-oxidant and antioxidant defensive mechanism in the vital organs as gill and liver of the euryhaline Cichlid fish *Etroplus maculatus*.

On the other hand, the present study also showed a significant decrease in the marker enzyme, alkaline phosphatase in gill and liver of chlordecone-treated fish. Alkaline phosphatase serves as diagnostic tool to assess the toxicity stress of chemicals in the living organisms (Harper, 1991). It is a hydrolytic lysosomal enzyme and is released by the lysosomes for the hydrolysis of foreign material. Subsequently the enzyme activity may begin to drop either as a result of having partly or fully encountered the toxin or as a result of cell damage. Alkaline phosphatase is also involved in the mediation of membrane transport and transphosphorylation. A decreased alkaline phosphatase activity in gill and liver of treated fish indicate the decreased state of inter and intracellular membrane transport and possibly this could be due to the toxicity of chlordecone.

CONCLUSIONS

There are considerable information from several studies indicating that environmental toxicants are responsible for many adverse effects in fishes and other animals. The present findings consequently prove that oxidative imbalance in gill and liver of treated fish could be due to the toxicity of chlordecone in the exposed fish.

ACKNOWLEDGEMENTS

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